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CspR, a Cold Shock RNA-Binding Protein Involved in the Long-Term Survival and the Virulence of *Enterococcus faecalis*

Charlotte Michaux,[a] Cecilia Martini,[a,c] Koki Shioya,[d] Sandra Ahmed Lecheheb,[e] Aurélie Budin-Verneuil,[a] Pascal Cosette,[e] Maurizio Sanguinetti,[c] Axel Hartke,[a] Nicolas Verneuil,[a] and Jean-Christophe Giard[b]

Unité de Recherche Risques Microbiens (U2RM), Equipe Stress Virulence[a] and Equipe Antibio-résistance,[b] Université de Caen, Caen, France; Institute of Microbiology, Catholic University of Sacred Heart, Rome, Italy[c]; Department of Bioengineering, Nagaoka University of Technology, Nagaoka, Japan[d]; and UMR 6270 CNRS, Proteomic Platform, Université de Rouen, Mont-Saint-Aignan, France[e]

By coprecipitation, we identified RNA-binding proteins in the Gram-positive opportunistic pathogen *Enterococcus faecalis* known to be deficient of the RNA chaperone Hfq. In particular, we characterized one belonging to the cold shock protein (Csp) family (Ef2925) renamed CspR for cold shock protein RNA binding. Compared to the wild-type strain, the Δ*cspR* mutant was less virulent in an insect infection model (*Galleria mellonella*) and exhibited a decreased persistence in mouse kidneys and a low survival rate in peritoneal macrophages. As expected, we found that the Δ*cspR* mutant strain was more impaired in its growth than the parental strain under cold conditions and in its long-term survival under nutrient starvation. All these phenotypes were restored after complementation of the Δ*cspR* mutant. In addition, Western blot analysis showed that CspR was overexpressed under cold shock conditions and in the stationary phase. Since CspR may act as an RNA chaperone, putative targets were identified using a global proteomic approach completed with transcriptomic assays. This study revealed that 19 proteins were differentially expressed in the Δ*cspR* strain (9 upregulated, 10 downregulated) and that CspR mainly acted at the posttranscriptional level. These data highlight for the first time the role of the RNA-binding protein CspR as a regulator in *E. faecalis* and its requirement in stress response and virulence in this important human pathogen.

Recently, the number of bacterial products whose expression depends on posttranscriptional regulation has increased significantly. This is due to the discovery of global posttranscriptional regulators (36), such as the Hfq RNA-binding protein (7, 14, 52, 53) or Csp (cold shock protein) (17, 22, 25), that can modulate the translation and stability of cellular mRNAs.

Like most of the RNA-binding proteins that are usually small-sized molecules (80 to 130 amino acids [aa]), Csp proteins, composed of about 70 amino acids (38), are, by far, the most strongly induced proteins at low temperatures (19). Several Csp homologues (from two to nine) (55) are present in Gram-positive and Gram-negative bacterial genomes, and a multiple-deletion analysis showed that Csp proteins play important roles not only during cold shock adaptation but also during the stationary phase or under nutrient stress (21). For *Bacillus subtilis*, Csp proteins have been shown to be essential for efficient adaptation to low temperatures and survival during the stationary phase (18). Moreover, a *cspB* mutant of *Staphylococcus aureus* exhibited an important growth defect, reduction in pigmentation and in resistance to certain antimicrobials (10). It has been proposed that the Csp proteins act as RNA chaperones, facilitating gene expression at low temperatures as transcriptional activators (3, 27, 29), transcriptional antiterminators (1), and/or as alternative translation initiation factors (21). The Csp regulatory activity is mainly at the posttranscriptional level with effects on the mRNA (39). The proteins limit the formation of secondary structure, enhancing the RNA lifetime by increasing its stability (11). In addition, these RNA-binding proteins facilitate the translation initiation because of the increased availability of the Shine-Dalgarno sequence (32).

The ubiquitous Gram-positive bacterium *Enterococcus faecalis* is a commensal of the intestinal tract in humans and a major opportunistic pathogen responsible for nosocomial infections (54). This "Janus face" bacterium is able to develop efficient adaptation processes to cope with environmental changes (37). Actually, about a dozen putative virulence genes have been reported in *E. faecalis*, including several transcriptional regulators (23, 30, 34, 41, 47). Among them, the sole example of an RNA-dependent posttranscriptional control involved in commensalism and virulence described so far concerns the utilization of ethanolamine (13). Note that, as for other Gram-positive bacteria such as *Streptococcus* species, *E. faecalis* does not contain any obvious Hfq homologue-encoding genes (49).

In the present study, we identify one RNA-binding protein, Ef2925 (renamed CspR for cold shock protein RNA binding), in *E. faecalis*. Using different infection models, we found that *cspR* plays a role in the virulence of *E. faecalis* and in the organ colonization and survival in macrophages. We also report the functional analysis of this RNA-binding protein, with the implication of *cspR* in the cold shock response and long-term survival. In addition, global proteomic and transcriptomic analyses allowed us to identify CspR putative targets.

MATERIALS AND METHODS

Bacterial strains, plasmids, growth conditions, and survival experiments. Bacterial strains and plasmids used in this study are listed in Table S1 in the supplemental material. Mutants were constructed from the pa-

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Address correspondence to Jean-Christophe Giard, jean-christophe.giard@unicaen.fr.
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rental *E. faecalis* strain EryS, an erythromycin-sensitive strain derivate from the vancomycin-resistant clinical isolate V583 (43). *Escherichia coli* DH5α (23) was used as the recipient for cloning. *E. faecalis* EryS and its derivates were grown, without shaking, at 37°C in M17 medium supplemented with 0.5% glucose (GM17). For the stationary-phase survival assay, T_0 was considered when the cultures reached an optical density at 600 nm (OD_{600}) of 1.5. CFU were determined by plating on GM17 and counting colonies after 24 h of incubation at 37°C. Strain survival is the ratio of the CFU at the different sampling times to the numeration at T_0. Each survival experiment was performed, at least, in triplicate. Growth of the *E. faecalis* strains was also examined in 40% horse serum and human urine for growth under oxidative stress conditions (shaking in GM17 medium supplemented with 1.5 mM H_2O_2 or in CCM17 MOPS [morpholinepropanesulfonic acid] medium supplemented with 0.5% glycerol). *E. coli* strains were cultured, with shaking, at 37°C in LB medium with chloramphenicol (10 μg/ml) when required. The antibiotic resistance of the wild-type and the *cspR* mutant strain was determined by the disk diffusion method. The usual enterococcal antibiogram including streptomycin, ampicillin, levofloxacin, kanamycin, erythromycin, lincomycin, pristinamycin, chloramphenicol, doxycycline, vancomycin, teicoplanin, sulfamethoxazole-trimethoprim, linezolid, rifampin, tigecycline, and gentamicin was performed. Results were interpreted as recommended by the Antibiogram Committee of the French Society for Microbiology (www.sfm-microbiologie.org).

General molecular methods. PCR was performed with Phusion high-fidelity DNA polymerase (Finnzymes, Vantaa, Finland). The primers used for this work are listed in Table S2 in the supplemental material. PCR products and plasmids were purified using the NucleoSpin plasmid kit (Macherey-Nagel, Dünen, Germany). Restriction endonucleases and T4 DNA ligases were purchased from Promega (Madison, WI) and used according to the manufacturer's instructions. Genomic DNA extraction and other standard techniques were carried out as described by Sambrook et al. (44).

Genetic construction and complementation of Δ*cspR* strain. For the construction of the Δ*cspR* deletion mutant strains, allelic replacements were carried out as described previously (5). For the Δ*cspR* strain, deletion occurred from 61 bp after the ATG start codon to 57 bp after the TAA stop codon. Briefly, DNA fragments (obtained using PCR with chromosomal DNA of *E. faecalis* EryS as the template) containing ligated upstream and downstream sequences (around 900 bp) of the desired deletion were cloned into the plasmid pLT06 (50) (see Table S1 in the supplemental material), and 1 μg of a recombinant plasmid was used to transform competent cells. Single-crossover transformants (dark blue colonies that are chloramphenicol resistant) were used for temperature shifts in order to release the plasmid. Candidate clones resulting from a double-crossover event were isolated on GM17 agar with or without chloramphenicol. Antibiotic-susceptible clones were analyzed for the presence of deleted *cspR* genes and verified by sequencing. For complementation construction, the pLT06wt*cspR* plasmid (containing the complete gene fragment) was introduced into the Δ*cspR* competent cells. Double recombination events allowed for the restoration of the intact *cspR* gene.

RNA isolation and qRT-PCR. RNAs were extracted by the method described by Toledo-Arana et al. (51). Briefly, cell pellets from a culture of 100 ml were taken over by 200 μl of Max bacterial enhancement reagent (Invitrogen, Carlsbad, CA). Next, the cell suspension was placed in a microtube containing 200 μl of RNase-free water and about 100 μg of glass beads and broken by two 30-s treatments in a FastPrep instrument (MP Biomedical LLC, Santa Ana, CA). After centrifugation, the aqueous phase was transferred into a microtube containing 1 ml of TRIzol (Invitrogen), mixed, and incubated at room temperature for 5 min. Cold chloroform was then added, mixed, and incubated for 3 min at room temperature. After centrifugation, the aqueous phase was mixed with 200 μl of cold chloroform. The mixture was then centrifuged, and the RNA contained in the aqueous phase was precipitated by the addition of isopropanol. The RNA pellet obtained by centrifugation was finally washed with ethanol and then dried at room temperature and resuspended in RNase-free water.

For reverse transcriptase PCR (RT-PCR) experiments using samples from wild-type or Δ*cspR* cells, 2 micrograms of RNA was reverse transcribed with random hexamer primers and QuantiTect enzyme (Qiagen, Valencia, CA) according to the manufacturer's recommendations. cDNA and genomic DNA (positive control) were then used as the templates for PCRs using the primers listed in Table S2 in the supplemental material and designed from the V583 genome sequence and with Primer3 software (http://frodo.wi.mit.edu/). Quantification of 23S rRNA and *gyrA* (encoding the A subunit of the DNA gyrase enzyme) mRNA provided internal controls. Amplification (using 5 μl of a 1/100 cDNA dilution), detection (with automatic calculation of the threshold value), and real-time analysis were performed in triplicate using the iCycler iQ detection system (Bio-Rad, Hercules, CA). Relative mRNA levels of each gene in each sample were calculated using the comparative cycle time as described previously (33).

Protein extractions and Western blotting. For total protein extractions, *E. faecalis* cells were harvested from cultures in exponential (OD_{600} of 0.5) or stationary growth phase (after 24 h of growth) in GM17 medium at 37°C or at 9°C ± 1°C. They were washed with a solution containing 50 mM Tris (pH 7.5), 50 mM Na_2SO_4, and 15% glycerol, resuspended in the same solution, and broken by shaking twice for 30 s in a FastPrep instrument (MP Biomedical LLC). Unbroken cells were removed by centrifugation (10,000 rpm for 10 min at 4°C). For electrophoresis, proteins were mixed with Laemmli buffer (28), heated for 5 min at 95°C, and separated by SDS-PAGE.

For Western blotting, after electrophoresis, proteins were transferred onto a polyvinylidene difluoride membrane which was then stained with Coomassie blue in order to verify that equal amounts of protein were present in all lanes. Blocking, incubation with antiserum against His-tagged CspR, and enhanced chemiluminescence detection (ECL detection kit; GE Healthcare, Little Chalfont, United Kingdom) were carried out as described previously (42).

Selection and identification of RNA-binding proteins. RNA-binding proteins were selected using Dynabeads oligo$(dT)_{25}$ (Invitrogen) according to the manufacturer's instructions, with the following modifications. Sixty micrograms of total RNA (mixture of 30 μg from exponential-phase cells and 30 μg from stationary-phase cells) was treated with poly(A) polymerase (Epicentre, Madison, WI) at 37°C for 30 min. Poly(A)-tailed RNA was mixed and annealed with Dynabeads oligo$(dT)_{25}$ by continuous rotation for 5 min at room temperature. The complex was washed three times with buffer 1 (10 mM Tris-HCl, 0.15 M lithium chloride [LiCl], 1 mM EDTA [pH 7.5]). Then, 1 mg of total protein extract (mixture of 500 μg from exponential-phase cells and 500 μg from stationary-phase cells) was added to the bead-RNA complex and annealed with RNA by continuous rotation for 10 min at room temperature. The bead-RNA-protein complex was washed twice with buffer 1 and buffer 2 (10 mM Tris-HCl, 0.5 M LiCl, 1 mM EDTA [pH 7.5]). The complex was resuspended in 10 mM Tris-HCl (pH 7.5), and the RNA-protein complex was removed from the beads by heat treatment.

Construction of *E. coli* strain M15/pQE$_{30}$*cspR* overproducing CspR. In order to produce antibodies against *E. faecalis* CspR, this protein was first overproduced. The corresponding gene was amplified using primers listed in Table S2 in the supplemental material and inserted downstream of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible promoter into the pQE$_{30}$ expression vector (QIAexpressionist kit; Qiagen) to obtain an N-terminal His_6-tagged recombinant protein. The resulting construction was electroporated into the *E. coli* M15pRep4 strain, creating the M15/pQE$_{30}$*cspR* strain (see Table S1 in the supplemental material). Recombinant CspR protein was overproduced and purified as described by Muller et al. (35).

Production of anti-CspR antibodies. The purified recombinant protein CspR was injected with a complete Freund adjuvant into a New Zealand white rabbit (42). After 3 injections (one every 2 weeks), serum was

collected and tested for anti-CspR antibodies by Western blotting with purified recombinant CspR.

Growth competition assays. Isogenic strains carrying either the wild type or a Δ*cspR* mutation were competed against each other. An equal quantity of each strain at an OD_{600} of 0.05 was diluted into the same flask containing 10 ml of fresh GM17 and allowed to compete for the same pool of nutrients at 37°C. At 2 and 3 h, colonies were obtained by plating appropriate dilutions on GM17 plates. For each time point, 100 colonies were tested by PCR amplification with the appropriate primers flanking the gene deletion (see Table S2 in the supplemental material) in order to determine the percentages of the wild-type and the Δ*cspR* strains. Each competition assay was performed at least three times.

Infection and macrophage survival experiments. Infection of *G. mellonella* larvae with *E. faecalis* was accomplished as described previously by Lebreton et al. (30). Briefly, using a syringe pump (KD Scientific, Holliston, MA), larvae (about 0.3 g and 3 cm in length) were infected subcutaneously with the washed *E. faecalis* wild-type strain, the Δ*cspR* mutant strain, and the complemented strain from an overnight culture in GM17, with $6 \times 10^6 \pm 0.6 \times 10^6$ CFU per larva administered in 10 μl of sterile saline buffer. In each test, 15 insects were infected, and the experiments were repeated at least five times. Larval killing was then monitored at 18 h postinfection.

The virulence of the wild-type strain and that of the *cspR* deletion mutant were also assessed in an intravenous infection model, essentially as described by Gentry-Weeks et al. (15). Briefly, overnight cultures of the strains grown in brain heart infusion (BHI) broth supplemented with 40% heat-inactivated horse serum were centrifuged, and the resulting pellets were resuspended in sterile phosphate-buffered saline (PBS) to achieve final concentrations of 1×10^9 bacteria/ml. Aliquots of 100 μl from each strain were used to inject each of 10 female BALB/c mice (10 weeks old; Harlan Italy S.r.l., San Pietro al Natisone, Udine, Italy) transurethrally. Infection experiments were repeated three times. Mice were monitored twice daily and were euthanized by CO_2 asphyxiation 7 days after infection. Kidneys were then removed aseptically, weighed, and homogenized in 5 ml of PBS using a stomacher (model 80; Pbi International, Milan, Italy) for 120 s at high speed. For CFU determination, serial homogenate dilutions were plated onto *Enterococcus* selective agar (Fluka Analytical, Switzerland) to avoid environmental contamination, and plating efficiency was comparable to that with a nonselective medium. CFU counts were analyzed by the unpaired *t* test.

Survival of *E. faecalis* in mouse peritoneal macrophages was also tested as described previously (16). Briefly, the cells were grown as standing cultures at 37°C in BHI for 16 h. The bacteria were then pelleted and resuspended in 5 ml of PBS for injection. Mice were infected with 10^7 to 10^8 cells of each strain (estimated by CFU determination) by intraperitoneal injection of 200 μl of the PBS-enterococcus suspension. After 6 h of infection, the peritoneal macrophages were collected by two peritoneal washes, each using 5 ml of PBS.

The cell suspension was dispensed into 24-well tissue culture plates and incubated at 37°C under 5% CO_2 for 2 h. After exposure to antibiotics (vancomycin at 10 μg/ml and gentamicin at 150 μg/ml) to kill extracellular bacteria (verified by the absence of CFU in supernatant fluids), the infected macrophages were washed, and triplicate wells of macrophages were lysed with detergent to quantify viable intracellular bacteria. No difference in the MICs of these antibiotics was observed for the Δ*cspR* and wild-type strains. The procedure was repeated three times, and results were analyzed using one-way analysis of variance with a Bonferroni correction posttest with SPSS statistical software (SPSS, Chicago, IL). All statistical analyses were performed using Prism software (version 5.00) for Windows (GraphPad Software, San Diego, CA). For all comparisons, a *P* value of less than 0.05 was considered significant.

Mass spectrometry and protein quantification using Progenesis liquid chromatography-mass spectrometry (LC-MS). After sampling and immediate cooling, all the bacterial pellets harvested in mid-exponential growth phase at 37°C were brought to room temperature for further treatment. First, bacteria were incubated twice for 10 min at 37°C and for 10 min at −80°C successively and subsequently submitted to four cycles of 30-s sonication in a lysis buffer (7 M urea, 2 M thiourea, 0.1% amidosulfobetaine [ASB14], 2 mM tributyl phosphine [TPB], 0.5% [vol/vol] carrier ampholytes [pH 3 to 10; Sigma]). Proteins extracts were then harvested in the supernatant after elimination of cellular debris by centrifugation (10,000 × *g* for 30 min at 4°C). Protein concentrations were evaluated using the Bio-Rad protein assay. Accordingly, 10 or 25 μg was loaded on 7% polyacrylamide gels using the Protean II xi vertical system (Bio-Rad), allowing for a short period of migration (1 h 30 min) in a stacking gel. After staining, the revealed protein band was excised and the proteins within the bands were reduced in 5 mM dithiothreitol and cysteines were irreversibly alkylated in 25 mM iodoacetamide. After washing with water, gel bands were submitted to protein digestion (trypsin from Promega, 1 μg per band). Several steps of peptide extraction were then performed in H_2O-CH_3CN-trifluoroacetic acid (TFA) mixtures (50:50:1), and the peptide fractions were combined and evaporated with a SpeedVac (Thermo Fisher Scientific, Waltham, MA).

The peptide extracts were reconstituted in the chromatography starting elution buffer (0.1% formic acid in water) at a concentration of 0.2 μg/μl. Five microliters was injected on a nanoscale liquid chromatography (nanoLC) system (Easy-nLC; Proxeon) coupled to a LTQ Orbitrap hybrid mass spectrometer (Thermo Fisher Scientific).

Raw data were then imported in Progenesis LC-MS software (version 4.0.4441.29989; Nonlinear Dynamics). After the feature alignment (typically around 50,000 features), the filtering of highly charged states (>8), and the application of a standard normalization procedure, peptide features with *P* values of <0.05, q values of <0.05, and a power value of >0.8 were kept for the identification step. The feature inclusion criteria for creating a peak list of MS-MS spectra were restricted to the top-10-ranked MS-MS data for the same feature. The merged peak list was searched against the *E. faecalis* V583 database using a local version of Mascot (version 2.2; Matrix Science, United Kingdom), allowing one missed cleavage and 5-ppm/0.6-Da mass deviations in MS and MS-MS data, respectively.

RESULTS

Identification of RNA-binding proteins in *E. faecalis* strain. We prepared RNA and protein samples from both exponential- and stationary-phase cells in order to increase the probability of obtaining RNA-associated proteins of *E. faecalis*. After the coprecipitation of total RNAs and total protein extracts using the Dynabeads oligo$(dT)_{25}$ (Invitrogen), the RNA-protein complex was removed from the beads by heat treatment. The elution sample containing the RNA-targeted proteins was analyzed by mass spectrometry for identification. Data revealed the presence of 4 proteins: a hypothetical protein (Ef0127), elongation factor Tu (Ef0201), a glyceraldehyde-3-phosphate dehydrogenase (Ef1964), and a cold shock domain-containing protein (Ef2925). According to the literature description of cold shock proteins that bind RNAs, we focused on Ef2925.

Amino acid sequence alignments revealed that Ef2925 shares between 68 and 92% homologies with the cold shock proteins present in major Gram-positive or -negative bacteria, including most virulent species such as *Bacillus anthracis*, *Yersinia pestis*, and *Clostridium botulinum* (Fig. 1). Specific conserved amino acid residues, RNA-binding motifs RNP-1 and RNP-2, which have been shown to be critical for full function of the Csp, are also present in the Ef2925 sequence.

To date, the whole genome sequences of 58 *E. faecalis* strains are available in the NCBI database (http://www.ncbi.nlm.nih.gov/). We performed standard BLAST analysis and detected the presence of *ef2925* in all the different *E. faecalis* strains, showing that this gene is part of the core genome. It is interesting to note

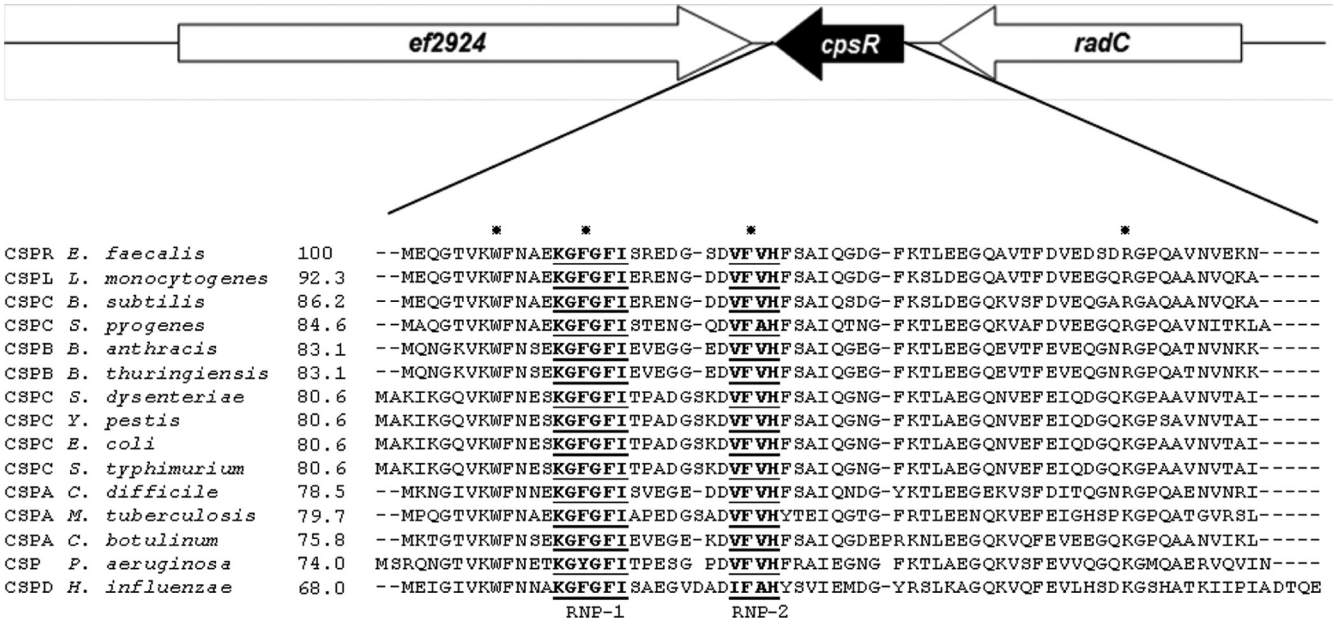

FIG 1 Genetic organization of the *cspR* (*ef2925*) chromosomal region of *E. faecalis* and sequence alignment of the cold shock domain proteins from different species. Large arrows represent the genes which compose the adjacent genetic regions of *cspR*, and their orientation shows the transcriptional direction. The numbers correspond to the percentage of homologies with CspR for *E. faecalis*. Conserved nucleic acid-binding sequence motifs, the RNA-binding motifs RNP-1 and RNP-2, are in bold and underlined. Conserved DNA-binding sites are indicated by a dash. The GenBank identifiers for these sequences are as follows: CspR of *Enterococcus faecalis*, AAO82613.1; CspL of *Listeria monocytogenes*, CAC99442.1; CspC of *Bacillus subtilis*, CAB12319.1; CspC of *Streptococcus pyogenes*, AAL98586.1; CspB of *Bacillus anthracis*, AAP27350.1; CspB of *Bacillus thuringiensis*, AAT61483.1; CspC of *Shigella dysenteriae*, AAG03845.1; CspC of *Yersinia pestis*, CAC90565.1; CspC of *Escherichia coli*, AAG56812.1, CspC of *Salmonella enterica* serovar Typhimurium, AAG56812.1; CspA of *Clostridium difficile*, YP_001087366.1; CspA of *Clostridium botulinum*, ABS38232.1; CspA of *Mycobacterium tuberculosis*, ABQ75472.1; Csp of *Pseudomonas aeruginosa*, AAG03845.1; and CspD of *Haemophilus influenzae*, AAX88488.1.

that *ef2924*, located upstream of *ef2925* in the *E. faecalis* genome, is annotated as encoding a metallo-beta-lactamase protein that presents a strong homology with the RNase RnjA of *Bacillus subtilis*. Located downstream of *ef2925*, *ef2926* encoded RadC, with a role in DNA repair and recombination.

Ef2925 is important for the cold shock response. As mentioned previously, Ef2925 harbors motifs of cold shock proteins. We then decided to test the response of the Δ*ef2925* mutant to a temperature downshift and the induction of the Ef2925 protein at 9°C ± 1°C. As shown in Fig. 2a, the evolution of OD_{600} values of the *E. faecalis* wild-type and Δ*ef2925* cells at 9°C ± 1°C were different. Indeed, after 48 h at 9°C, the wild-type strain and the Δ*ef2925* mutant strain reached OD_{600}s of 1.1 and 0.6, respectively (Fig. 2a). The complemented mutant restored the wild-type behavior (Fig. 2a).

In order to know if Ef2925 is cold shock inducible, a Western immunoblot analysis was performed with anti-Ef2925 antibody (Fig. 2b). The quantity of this protein obviously increased after 4 h of incubation at 9°C ± 1°C (lane 3) and even more after 8 h (lane 4) compared to the amount observed when the cells were in the exponential phase at 37°C (lane 2). This experiment was also used to confirm the absence of the Ef2925 protein in our Δ*ef2925* mutant strain (lane 1). Based on these data, Ef2925 was named CspR for cold shock protein RNA-binding protein.

CspR plays a role in stationary-phase survival and bacterial competition. Because of the role of cold shock protein described in the literature (25), we were interested to show the effect of a *cspR* mutation on cellular survival during the stationary phase of *E. faecalis*. The growth of the Δ*cspR* strain and that of the wild-type

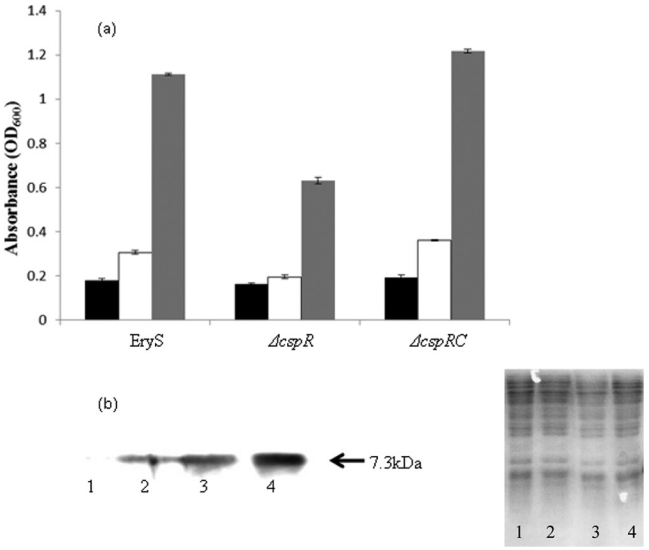

FIG 2 Effect of CspR during cold shock stress. (a) OD_{600} values of the Δ*cspR* mutant strain, the wild-type (EryS) strain, and the complemented (Δ*cspRC*) strain grown in GM17 medium, without shaking, at 9°C ± 1°C at 10 h (black bars), 24 h (white bars), and 48 h (gray bars). The experiment was repeated at least four times, and the results represent the means ± standard deviations. (b) Analysis of the effect of cold shock stress at 9°C ± 1°C on the amount of CspR protein by Western immunoblotting. Each lane contains equal amounts of 10 μg of total protein (controlled by Coomassie blue staining shown on the right). Samples were obtained from cells harvested in the Δ*cspR* mutant strain (lane 1), the wild-type strain (lane 2), the wild-type strain after 4 h of incubation at 9°C ± 1°C (lane 3), and the wild-type strain after 8 h of incubation at 9°C ± 1°C (lane 4). The arrow indicates the molecular mass of the cross-reactive band in the wild-type strain.

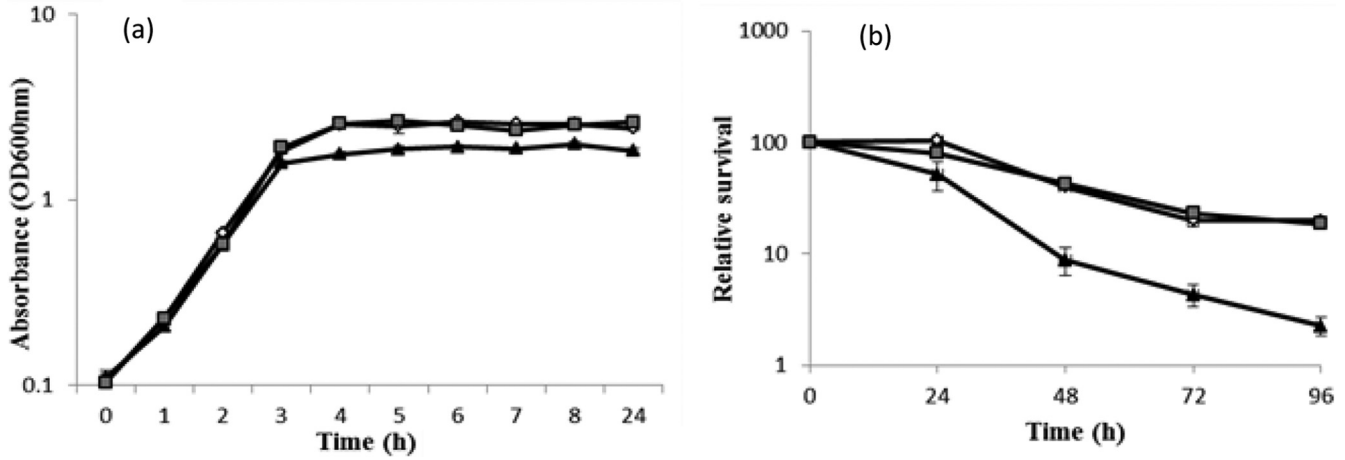

FIG 3 Effect of *cspR* inactivation on long-term survival. (a) The *E. faecalis* wild-type strain (white diamonds), Δ*cspR* (black triangle) mutant strain, and Δ*cspR* complemented strain (gray square) were grown in GM17 medium, without shaking, and optical density at 600 nm (OD_{600}) was determined. (b) Relative survival during stationary phase. The survival of the *E. faecalis* wild-type (white diamonds) strain, Δ*cspR* (black triangle) mutant strain, and complemented strain (gray square) was determined as the ratio between the number of cells at the onset of starvation (T_0) and the number of cells surviving after 24, 48, 72, and 96 h. The curves shown represent the average of, at least, three independent experiments and the results represent the means ± standard deviations.

strain at 37°C in GM17 medium, without shaking, were similar during the exponential phase. In contrast, the mutant strain revealed a slight but significant difference of the final OD_{600} during the stationary phase compared to the parental strain ($P = 0.002$) (Fig. 3a). Indeed, the wild-type culture reached the stationary phase at an OD_{600} of 2.42 versus 1.8 for the Δ*cspR* mutant cells. We then tested the ability of both strains to survive during long-term starvation. Cell count experiments were carried out for 96 h (Fig. 3b). Results showed that 48 h after the onset of starvation, about 60 and 90% of the cells from the wild type and the Δ*cspR* mutant were dead, respectively. Thereafter, at 72 h, the CFU count of the wild-type cells remained fairly constant for several days (data not shown), whereas almost no Δ*cspR* mutant cells survived after 96 h of starvation (Fig. 3b). The complemented mutant restored the wild-type growth and survival rates (Fig. 3b). We used the anti-CspR antibody to visualize the amount of CspR when *E. faecalis* was in the stationary phase after 24 h of growth. As shown in Fig. 4, CspR appeared obviously overexpressed in the cells harvested in starvation condition compared to bacteria in the exponential phase of growth.

Despite the fact that the Δ*cspR* and wild-type strains demonstrated the same growth rate and the same amount during the first 3 hours of growth, we studied the putative growth advantage of the wild-type strain compared to the Δ*cspR* mutant. The results of the competition experiments using a mixture of both strains inoculated at the same amount (50%/50%) are presented in Fig. 5. In this coculture, after 2 h of exponential growth, no difference was observed between the number of the wild type and the Δ*cspR* mutant: both types of cells remained present at the same amount. However, after 3 h of growth, the relative level of the wild-type cells was significantly higher (70%) than the Δ*cspR* mutant strain (30%) (Fig. 5).

CspR is involved in virulence and bacterial persistence within mouse organs and peritoneal macrophages. Since the RNA chaperone may have a role in the virulence in different bacterial species (7), we analyzed the involvement of the identified RNA-binding protein CspR in the pathogenicity of *E. faecalis*. Then, we compared the abilities of the wild-type and the corresponding mutant strains to kill *Galleria mellonella* larvae. Note that the bacterial cells used to infect larvae were from overnight

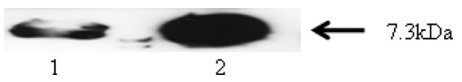

FIG 4 Analysis of the amount of the CspR protein by Western immunoblotting. Each lane contains 10 μg of the total proteins extracted from of the wild-type strain harvested in the exponential phase (lane 1) and the stationary phase (lane 2). Arrow indicates the molecular mass of the cross-reactive band in the wild-type strain.

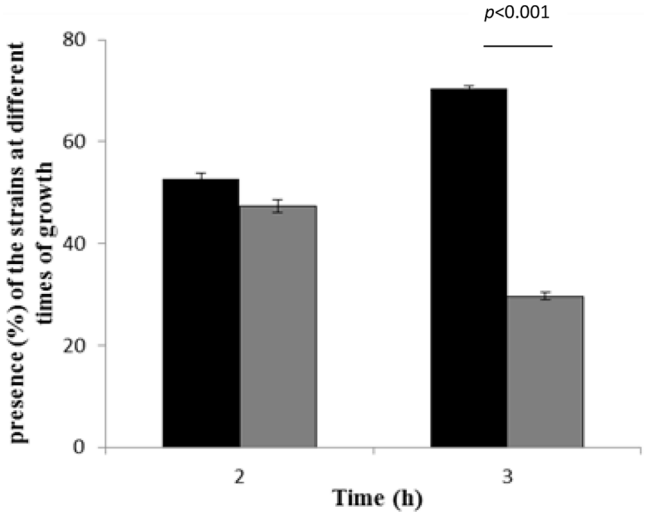

FIG 5 Competition assays of the Δ*cspR* strain (gray bars) competed with the wild-type strain (black bars) when the two strains were incubated together. PCR amplifications with appropriate primers flanking the gene deletion were performed on 100 colonies in order to discriminate the wild-type and Δ*cspR* mutant strains. Bars represent the average of, at least, three independent experiments and the results represent the mean ± standard deviation.

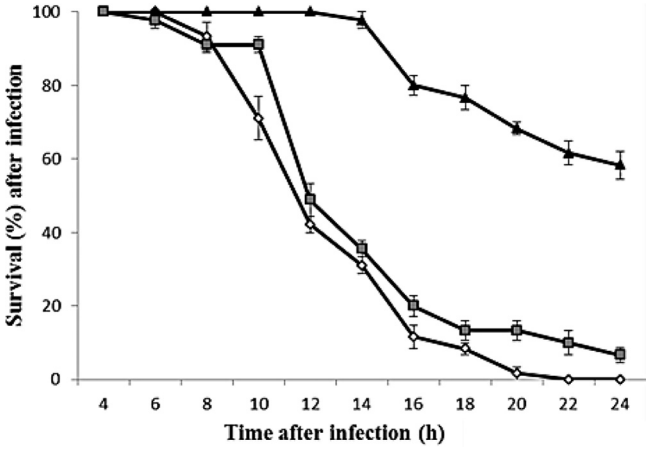

FIG 6 Effect of the Δ*cspR* inactivation on virulence. Percentage of survival of *G. mellonella* larvae at 18 h after infection, with approximately 1.2×10^5 CFU of the *E. faecalis* wild type (EryS) (white diamonds), Δ*cspR* mutant (black triangles), and Δ*cspR* complemented strain (grey squares). Experiments were repeated at least three times, and the results represent the means ± standard deviations for live larvae.

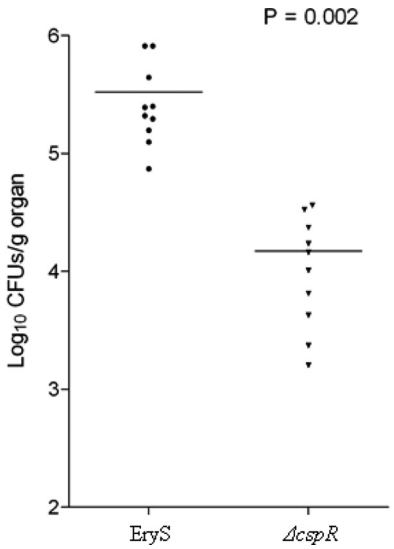

FIG 7 Bacterial persistence within mouse kidneys. Enterococcal tissue burdens in kidneys from BALB/c mice infected intravenously with 1×10^8 cells of the *E. faecalis* wild-type (EryS) and Δ*cspR* mutant strains are shown. The results represent values recorded separately for each of the 10 mice. Horizontal bars represent the geometric means. *P* values of less than 0.05 were considered to be significant.

cultures with equal numbers of CFU. As shown in Fig. 6, the rate of killing was significantly lower in the larvae infected with the Δ*cspR* mutant strain than in those infected with the wild type. When the animals were infected with wild-type cells, dead larvae were observed after 8 h, whereas when the Δ*cspR* mutant cells were infected, dead larvae were observed after 14 h (Fig. 6). After 24 h of infection, all of the wild-type-infected larvae were killed, whereas 58% of the ones infected with the Δ*cspR* strain survived ($P < 0.001$) (Fig. 6). The nonpathogenic Gram-positive bacterium *Lactococcus lactis* strain IL1403 was used as a control. All the larvae infected with this microorganism survived throughout the experiment (data not shown). Assays conducted with the complemented Δ*cspR* mutant strain, in which the virulence was restored to the wild-type level, confirmed that the observed phenotype was due to the deletion of *cspR* (Fig. 6). We then examined the fates of the Δ*cspR* mutant and wild-type strains in the murine systemic infection model, specifically, tissue burdens in kidneys of infected mice. As shown in Fig. 7, the Δ*cspR* strain exhibited a decrease of 1.5 log units ($P = 0.002$) in kidney compared to the parental strain, showing that CspR is involved in the survival of *E. faecalis* within the host. It is interesting to note that the growth of the Δ*cspR* mutant and that of the parental strain in urine and horse serum were identical (data not shown).

The phagocytic cells constitute an important part of the innate immunity against pathogens. To determine whether the Δ*cspR* strain was affected at this stage of infection, the cells were tested in an *in vivo-in vitro* macrophage infection model. The intracellular survival of the Δ*cspR* and wild-type strains was monitored by determining the number of viable bacteria inside mouse peritoneal macrophages over a 72-h time course. No significant difference was observed between the cell counts at 8 h postinfection, suggesting that levels of phagocytosis of the two strains by the macrophages were similar. However, as shown in Fig. 8, at 24, 48, and 72 h postinfection, the Δ*cspR* strain was significantly intracellularly less present. At 72 h after infection, the survival of the Δ*cspR* bacteria in mouse macrophages was approximately 2 log units less than the wild-type strain (Fig. 8). This difference does not seem to be due to a putative effect of oxidative stress conditions. Indeed, the *cspR* inactivation during growth under H_2O_2 or with glycerol as the sole carbon source has been tested and no obvious phenotype has been observed. The environmental conditions are different *in vitro* and *in vivo*, but it is tempting to parallel the reduced survival in macrophages and the decreased ability to survive during starvation.

Identification of CspR putative targets. A comparison of the proteomic profiles of the wild-type and Δ*cspR* mutant cells was performed to identify the target candidates of the RNA-binding protein CspR. Total proteins were purified from cells harvested in the mid-exponential growth phase at 37°C. Polypeptides whose expressions differed by more than 2-fold were further analyzed

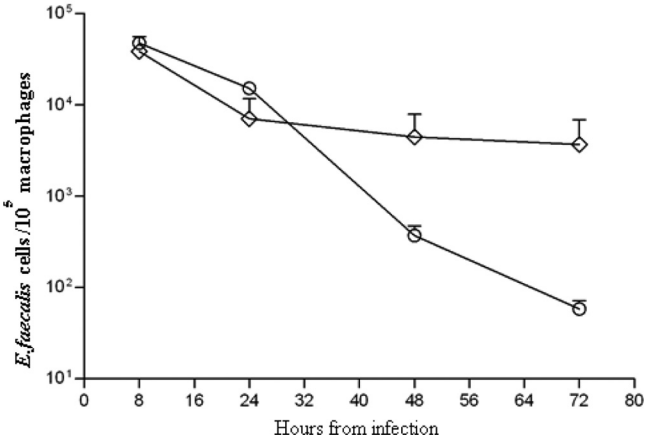

FIG 8 Time course of intracellular survival of the *E. faecalis* wild type (◇) and Δ*cspR* mutant (○) within murine peritoneal macrophages. The results represent the means ± standard deviations of viable intracellular bacteria per 10^5 macrophages from three independent experiments with three wells.

TABLE 1 Upregulated and downregulated proteins and expression of corresponding genes in the Δ*cspR* strain[a]

Target	Protein name	Function	Fold change in expression using:	
			Global proteomic approach	qRT-PCR approach
EF3115	Conserved hypothetical protein		+25.11	ND
EF0709 *(ptsH)*	Phosphocarrier protein HPr	PTS, signal transduction	+24.69	ND
EF0211 *(rplV)*	50S ribosomal protein L22	Ribosomal proteins: synthesis and modification	+13.60	ND
EF1217	Lipoprotein, putative	Cell envelope	+3.97	ND
EF1218	Spermidine/putrescine ABC transporter	Transport and binding protein, amino acids, peptides and amines	+2.46	ND
EF2100	Hypothetical protein		+2.07	+11
EF0633 *(tryS-1)*	Tyrosyl-tRNA synthetase	tRNA aminoacylation	+2.00	ND
EF0715 *(tig)*	Trigger factor	Protein folding and stabilization	+2.00	ND
EF1527	GTP-binding protein	Unknown	+2.00	ND
EF0323	Hypothetical protein		−2.56	+3.74
EF2939	Cold-shock domain family protein	Cellular process, adaptation to atypical conditions	−2.33	+2.5
EF2634 *(groES)*	Chaperonin, 10 kDa	Protein folding and stabilization	−2.27	ND
EF2847	Conserved domain protein		−2.22	ND
EF0312	Aspartate 1-decarboxylase domain protein	Unknown	−2.13	+4.22
EF2940	Hypothetical protein		−2.13	+3.46
EF1142	Hydrolase, haloacid dehalogenase-like family	Unknown enzyme	−2.08	ND
EF2904	Hypothetical protein		−2.08	ND
EF0355	Endolysin	Mobile and extrachromosomal element, prophage functions	−2.04	ND
EF2719 *(rplk)*	50S ribosomal protein L11	Ribosomal proteins: synthesis and modification	−2.00	ND

[a] Proteins and RNA samples were obtained from log-growth-phase cells. ND, no difference.

and listed as putative members of the CspR targets (Table 1; see also Table S3 in the supplemental material). This study revealed that 19 proteins were differentially expressed: 9 and 10 exhibited increased and reduced expression in the mutant strain, respectively. Among these, the protein Ef0709, which corresponds to the phosphocarrier protein HPr, was 24-fold more abundant in the Δ*cspR* mutant cells. Other proteins highly overexpressed in the absence of CspR included hypothetical protein Ef3115 and 50S ribosomal protein L22 Ef0211, with values of 25.11- and 13.60-fold, respectively. Note that the 10 polypeptides with reduced amounts in the mutant cells were only between 2- and 2.56-fold underexpressed (Table 1; see also Table S3). The global proteomic approach was completed by a transcriptomic assay of corresponding genes by quantitative RT-PCR (qRT-PCR). Among the 19 candidates, 14 showed no difference in transcriptional levels between the wild-type and mutant strains, arguing for a possible role of CspR in posttranscriptional regulation. For the protein Ef2100 overexpressed in the mutant (2-fold), the transcription of the corresponding gene also appeared much higher in the mutant than in the parental strain. The impact of the lack of CspR on the transcriptional and posttranscriptional regulation of *ef0312*, *ef0323*, and the *ef2939-ef2940* putative operon (*ef2939-2940*) appeared antagonistic: the levels of transcription of these loci were more important in the Δ*cspR* mutant than in the wild type, whereas the amount of detectable protein was less important (Table 1; see also Table S3).

We also performed qRT-PCR experiments on genes adjacent to *cspR* on the chromosome (*ef2924* and *ef2926*) and other genes encoding cold shock domain proteins of *E. faecalis* (*ef0781*, *ef1367*, and *ef2939*), but no relevant differences were observed (data not shown).

DISCUSSION

Several global regulators have recently been shown to act at the posttranscriptional level (36). This is the case for the RNA-binding protein Hfq, which is involved in regulation, fitness, and virulence of an increasing number of Gram-positive and -negative pathogens (4, 7). However, despite the important role of Hfq, no homologous protein is present in some genomes of low-GC-percent, Gram-positive lactic acid bacteria, such as *Streptococcus pyogenes*, *Lactococcus lactis*, and *E. faecalis* (49). In this context, the main goal of this work was to find RNA-binding proteins that may act as posttranscriptional regulators in *E. faecalis*, an important opportunistic pathogen. We identified Ef2925 (renamed CspR in this study), a member of the cold shock family harboring the highly conserved RNA-binding motifs RNP-1 and RNP-2, by screening the RNA-associated proteins analyzed by mass spectrometry (46). The cold shock proteins have already been shown to be able to destabilize mRNA secondary structures at low temperatures and act as an RNA chaperone (39). In some Gram-positive or -negative bacteria, RNA chaperones (26) are also involved in the cell division, the condensation of the chromosome and in several stress responses like increasing sensitivity to starvation, ethanol, high osmolarity, or antibiotics (6, 18, 27, 45, 55). Note that the usual enterococcal antibiogram on the Δ*cspR* mutant and wild-type strains did not show any difference of resistance. For example, in *E. coli* (56) and *B. subtilis* (21), Csp is induced during stationary phase and has a role in the starvation stress response. The overexpression of CspR observed in stationary phase and the important decrease of survival of the Δ*cspR* mutant during starvation revealed the role of the RNA-binding protein in this growth phase. As expected, CspR also played a role

in the cold shock stress response: the Δ*cspR* mutant was impaired in its growth at 9°C ± 1°C and CspR was overexpressed at low temperatures. Long-term survival and the cold shock response seem to have similarities. Both situations resulted in a rapid decrease in the translational capacity of the cell, equivalent to the treatment with inhibitors of translation, that is probably due to the reduced availability of functional ribosomes (18). All our results point to the importance of CspR for *E. faecalis*, including our cocultivation competition experiments with the wild type and the mutant that revealed the selective advantage CspR provides.

An important finding of this work is that the Δ*cspR* strain was less virulent than the parental strain in the *G. mellonella* model. The *Galleria* immune response has a number of structural and functional similarities with the innate immune response of mammals, and this insect model has been extensively used to evaluate the involvement of *E. faecalis* pathogenic factors in virulence (i.e., Fsr, GelE, Ace, and SlyA) (23, 30, 34, 47). In addition, the number of mutant cells recovered from macrophages and from kidney after systemic infections was lower than that of the wild type, which confirms the role of CspR in the pathogenicity of *E. faecalis*. Some RNA-binding proteins are known to modulate the transcription of virulence and/or stress genes. For example, enumeration of *L. monocytogenes* cells in organs of infected mice showed a clear reduction of the Δ*hfq* mutant compared to the wild type (9). To our knowledge, *vacB* of *Aeromonas hydrophila* encodes an exoribonuclease, a unique enzyme required for growth at low temperatures that has been shown to be involved in virulence (12). Our work is the first report with implications that a cold shock protein is involved in the virulence of Gram-positive bacteria. It can be suggested that the reduced virulence of the Δ*cspR* mutant may be due to the decreased ability of this strain to survive during long-term starvation.

A global proteomic approach is the most appropriate strategy to detect putative targets of RNA-binding proteins. Nineteen proteins have been shown to be differentially expressed when we compared the wild-type and Δ*cspR* mutant proteomic profiles. Transcriptomic analysis of the corresponding genes confirmed that this deregulation was due to the lack of CspR and operated mainly at the posttranscriptional level. In the Δ*cspR* mutant, 9 and 10 proteins were over- and underexpressed compared to the wild-type strain, respectively. This suggests that this RNA-binding protein could either increase or decrease the sensitivity of the corresponding mRNA according to the target. However, the most spectacular effects concerned proteins observed at a larger amount in the Δ*cspR* mutant. It is then tempting to speculate that CspR may participate in the mRNA degradation, such as described for Hfq. In this context, it is interesting to note that the gene *ef2924* (located just upstream of *cspR*) has 82% similarity with *rnjA* of *B. subtilis*, which encodes an RNase. The interconnection of this putative RNase, identified as RnjA, and CspR should be further investigated (24). The most upregulated (24.69-fold) target was the phosphocarrier protein HPr encoded by *ef0709* (*pstH*). HPr is involved in the phosphoenolpyruvate-dependent sugar phosphotransferase system (PTS) and implicated in the concomitant uptake and phosphorylation of carbohydrates. The cold induction of this protein has already been observed for *B. subtilis* (20) and *Lactobacillus sakei* (2). Interestingly, some of the putative CspR targets seem to have a role in the translation process and/or cold shock response. Two of them (*ef0211* and *ef2729*) encode 50S ribosomal proteins. In *E. coli*, the cold shock RNA helicase CsdA participates in the biogenesis of the 50S ribosomal subunit at low temperatures, probably by binding to and altering the RNA structure of the 50S precursor (8). Ef0715, encoded by *tig* and annotated as a trigger factor, was 2-fold induced in the Δ*cspR* mutant strain. This factor is a ribosome-associated chaperone that assists early folding steps of nascent proteins in bacteria, and it has been shown to be the major cold acclimation protein and the primary chaperone in the Antarctic bacterium *Pseudoalteromonas haloplanktis* (40). Note that the cochaperone GroES (Ef2634) was another CspR target involved in protein folding and stabilization. Ef1218, annotated as spermidine/putrescine ABC transporter and induced 2.46-fold in the mutant strain, may also have a link with the cold shock response since, in *E. coli*, the spermidine acetylation is increased in response to cold shock (31). None of the putative targets taken individually could fully explain the phenotypes observed for the Δ*cspR* mutant. However, our proteomic approach allows us to identify the very diverse cellular processes affected by the lack of CspR. Further studies to identify molecules interacting directly with CspR should be carried out.

We recently identified several small RNAs transcribed from the intergenic regions of the genome of *E. faecalis* and differentially expressed under stress conditions (48). It is very probable that their role in posttranscriptional regulation involved RNA-binding proteins that remain to be discovered. *E. faecalis* is deficient in the major RNA chaperone Hfq, so our work constitutes the first step toward the characterization of more specific RNA-binding proteins that act as posttranscriptional regulators and that are essential for the bacterial survival and virulence.

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